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AGE-DEPENDENT ACCUMULATION OF ADVANCED GLYCATION ENDPRODUCTS IS ACCELERATED IN COMBINED HYPERLIPIDEMIA AND HYPERGLYCEMIA, A PROCESS ATTENUATED BY L-ARGININE

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ABSTRACT

In this study we have investigated the occurrence of "classical" Amadori rearrangement products of AGE-proteins in the vascular mesenteric bed and in the lens of Golden Syrian hamsters (12 weeks old) rendered simultaneous hyperlipidemics-diabetics (HD), or hyperlipidemics (H) for 24 weeks. For the next 4 weeks the hamsters in HD and H groups received by gavage a solution of 3 mM L-arginine, with the intent to look for the potential effects of L-arginine on the fluorescence of tissular AGE-proteins. Age-matched normal hamsters were used as controls (C). The AGE-products of proteins, and the AGE-collagen isolated from the mesenteric bed were quantitated by fluorescence spectroscopy at ex: 370 nm/em: 440 nm. The results showed that: (i) compared to the fluorescence levels of AGE-proteins detected at C goup, in HD group the fluorescence of AGE-proteins was found 2.78 and 7.41 fold increased in the vascular mesenteric bed and lens, respectively; (ii) in H group the fluorescence of AGE-proteins was 2.36 fold augumented in the vascular mesenteric bed, and 5.43 fold in the lens (versus the C goup); (iii) the aging occurring during the 24 weeks of the experiment induced a small increase in AGE-proteins fluorescence in both mesentery (1.76 fold) and lens (3.83 fold), compared to the levels measured in C group at the inception of the study (12 weeks old hamsters); (iv) the fluorescence of AGE-proteins in the vascular mesenteric bed and in the lens of hamsters in HD and H groups correlated with the increase in circulating plasma glucose and cholesterol concentrations throughout the experiment; (v) L-arginine dietary supplementation in HD and H groups, diminished the AGE-collagen fluorescence in the mesentery to ~ 35% and ~ 17%, respectively; in the lens the fluorescence of AGE-proteins was reduced to 65-70% of the levels found in HD and H groups (at 24 weeks). This study showed for the first time that simultaneous hyperlipidemia-hyperglycemia induced an enhanced accumulation of fluorescent AGE-proteins in the mesentery and lens (comparatively to the effect of hyperlipidemia and of chronological aging monitored during the experiment), and that in vivo L-arginine administration decreased the fluorescence of tissular AGE-proteins (AGE-collagen included). The latter observation may bring another area of potential intervention in the adjunct efforts to find out inhibitors of AGE formation, and thus to reduce the increased levels of AGE-proteins accumulated in tissues when diabetes is additionally complicated with atherosclerosis.

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INTRODUCTION

The non-enzymatic protein glycation is a post-translational modification of proteins that starts by the covalent reaction between the sugar-derived carbonyls and the free amino groups in proteins. The process affects mostly long-lived proteins. In time, the initial Schiff base condensation products follow a complex cascade of chemical reactions performed autocatalitically (rings closures, condensations, crosslinkings, rearrangements, typical for the "classical" Amadori products), or oxidatively ("glycoxidation") leading to a class of heterogenous irreversible products collectivelly termed "Advanced Glycation Endproducts" (AGE)(1,2). Initially, the AGE-proteins were found to accumulate during aging (3, 4), and at an accelerated rate in diabetes. In the hyperglycemic milieu collagen, elastin, laminin, crystallin, and the myelin sheath proteins were modified by AGE adducts determining structural changes in tissues rich in these proteins (basal lamina, extracellular matrix of the vascular wall, lens) (5, 6, 7, 8). The formation of AGE-products was reported also in atherosclerosis: AGE modified the molecules of low density lipoprotein (LDL) (9) and of oxidized LDL (10), and were detected within vascular atheroma (11, 12, 13) where could originate also from the aldehydes generated by lipid peroxidation (14).

In the current study we examined the effect of simultaneous hyperlipidemia-hyperglycemia on the presence of AGE-proteins, and the influence of in vivo L-arginine administration (in combined hyperlipidemia-diabetes) two issues not investigated so far, but with potential relevance for accelerated diabetic atherosclerosis. The experiments were performed on Golden Syrian hamsters, an animal model suitable for inducing athero-

sclerosis (15), streptozotocin - diabetes (16), and simultaneous hyperlipidemia-hyperglycemia (17). In view of the enhanced and accelerated deleterious effects of hyperglycemia when hyperlipemia was additionally present (18) we have concentrated the attention on the vascular mesenteric bed (containing the resistance arteries involved in blood pressure regulation) and on the lens (a target of diabetic complications). The mesenteric vascular bed is known to accumulate AGE-collagen during advanced chronological age (19) and in diabetes, which caused also the hypertrophy of the mesenteric vasculature (20,21). The lens crystallin glycated in vitro showed a preferential glycation of αA crystallin subunits (6); in the diabetic (cataractous) lens crystallin accumulation of AGE-proteins was reported (22, 23, 24). In vivo treatment with L-arginine had benefic effects in diabetes reducing glomerular basement membrane N-carboxymethyllysine collagen, an AGE generated by "glycoxidation" (25). There are no data on L-arginine influence on AGE-proteins levels in hyperlipidemia, or in diabetes aggravated atherosclerosis.

From the complex glycation cascade we have investigated in this study the AGE "classical" Amadori rearrangement products, which share in common a peak of fluorescence occurring at 440 nm when excited at 370 nm that is used for their evaluation (6, 19, 26-30).

RESULTS

Characterization of animals

Normal hamsters

The body weight of normal hamsters from 6 to 48 weeks of age was in the range of 83.36 ± 17.28g. Plasma glucose concentration was 94.72 ± 16.98 mg/dl, and the cholesterol level was 75.30 ± 4.42 mg/dl.

Simultaneous hyperlipidemic-diabetic (HD) hamsters

The body weight of hamsters in HD group, measured during 2 to 24 weeks of induced hyperlipidemia and diabetes was 78.34 ± 19.58g. This represents an ~ 15% diminishment as compared to the body weight of age-matched animals in C group (92.17 ± 23.04g). Plasma glucose levels augumented from 108.72 mg/dl (at 2 weeks) to 265-300 mg/dl after 24 weeks of combined diseases. The cholesterol concentration in plasma enhanced gradually during the experiment, and reach after 24 weeks ~ 10 times the normal value.

Hyperlipidemic (H) hamsters

The body weight of hamsters in H group maintained in the normal range, i.e. 96.14 ± 22.79g throughout the experiment. The plasma glucose concentration varied slightly around the normal levels, while the cholesterol concentration increased steadily, and attained an ~ 9 fold the normal levels after 24 weeks of feeding the animals with the high fat diet.

Influence of age on AGE-protein fluorescence

The effect of aging on normal hamsters was followed for a time interval from 6 to 48 weeks, as depicted in

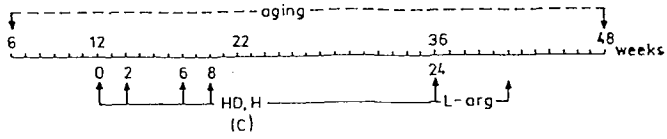

Fig.1: The time table of the experiments performed: (i) aging (for 6 to 48 weeks), (ii) concomitant hyperlipidemia - diabetes, and hyperlipidemia (installed in 12 weeks old hamsters for 24 weeks), and (iii) 4 weeks treatment with 3 mM L-arginine of the 24 weeks diseased hamsters.

Figure1. The animals gradually accumulated increasing amounts of fluorescent AGE-proteins in both vascular mesenteric bed and lens crystallin (Figure 2). Comparatively to young (6 weeks) hamsters, after 48 weeks of chronological age the augmentation of AGE-protein fluorescence was 2.50 fold enhanced in the mesentery (30.10 ± 1.50 vs. 11.92 ± 1.0 AU/μg protein, P=0.00079), and 11.60 fold increased in lens (3.50 ± 0.3 vs. 0.30 ± 0.1 AU/μg protein, P=0.00024).

The hamsters 12 weeks old were selected for the HD, H and C groups. At this biological age the AGE-protein fluorescence in the mesentery represented 12.50 ± 1.2 AU/μg protein, and in the lens 0.6 ± 0.1 AU/μg protein. The aging occurring during the 24 weeks of the experiment induced an increase in AGE-protein fluorescence in both mesentery (22.00 ± 1.4 AU/μg protein) and lens (2.3 ± 0.3 AU/μg protein).

Influence of hyperlipidemia-diabetes and hyperlipidemia on AGE-protein fluorescence

Vascular mesenteric bed

The diagram in Figure 2a shows the gradual increase (up to 2.78 fold above the level at the inception of the experiment) in the fluorescence of AGE-proteins during the 2 to 24 weeks of concomitant hyperlipidemia-diabetes (34.78 ± 0.8 vs 12.50 ± 1.2 AU/μg protein, P=0.00091), and a 2.36 fold enhancement, when the effect of hyperlipidemia was investigated (29.51± 0.78 vs 12.50 ± 1.2 AU/μg protein, P=0.00022). The age-matched control animals showed in the same time interval (12 to 36 weeks of age) an elevation of AGE-protein fluorescence of 1.76 fold (22 ± 1.4 vs 12.50 ± 1.2 AU/μg protein, P=0.00039).

Lens

Comparing to the level of AGE-protein fluorescence at the initiation of the experiment, after 24 weeks, the fluorescence of AGE-proteins was 7.41 fold increased in HD group (4.45 ± 0.3 vs 0.6 ± 0.1 AU/μg protein, P=0.0024), and 5.43 fold increased in H group (3.26 ± 0.3 vs 0.6 ± 0.1 AU/μg protein, P=0.0022) as represented by the results in Figure 2b. The control age-matched hamsters displayed in the same time interval (12 to 36 weeks of biological age) an augmentation of AGE-protein fluorescence of 3.83 fold (2.3 ± 0.3 vs 0.6 ± 0.1 AU/μg protein, P=0.0076).

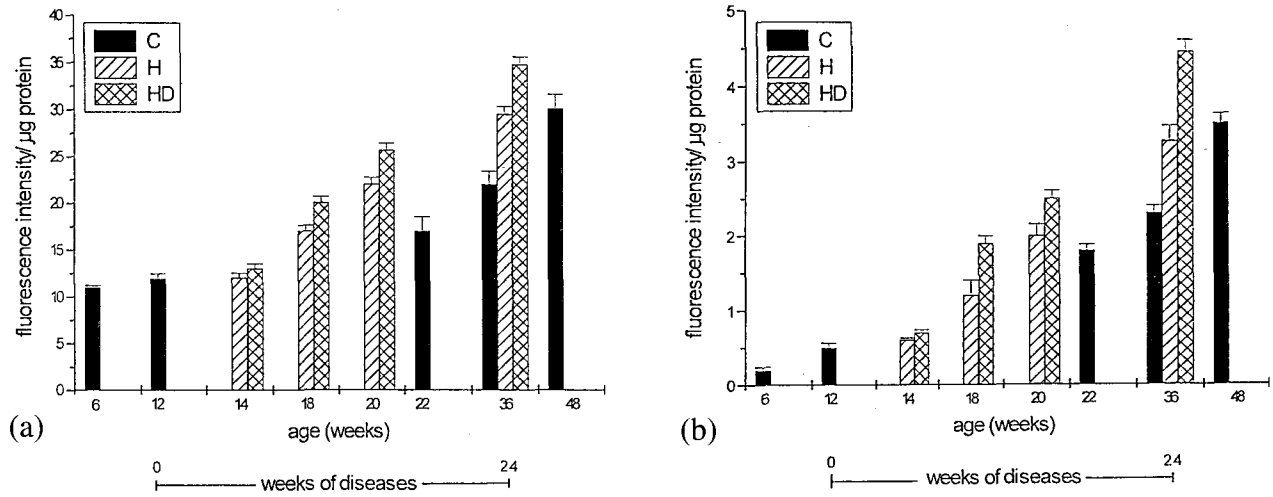

Fig. 2: AGE – protein fluorescence in the vascular mesenteric bed (a) and lens (b) of normal hamsters (C, aged from 6 to 48 weeks), of simultaneous hyperlipidemic-diabetic (HD) and hyperlipidemic (H) hamsters (at 2, 6, 8 and 24 weeks of disease/s).

Correlation between plasma cholesterol and glucose levels and the fluorescence of tissular AGE-*proteins*

The AGE-protein fluorescence in the mesentery was significantly associated with the increase in plasma glucose and cholesterol concentrations throughout the experiment (Figure 3 a,b) with increased levels in HD group vs.H. Similar plots were obtained for the lens AGE-protein fluorescence (Figure 3 c,d).

Effect of in vivo administration of 3 mM L-arginine

Body weight and plasma homeostasis

The in vivo administration of 3 mM L-arginine for 4 weeks improved the weight loss of hamsters in HD group with ~ 2.75%, while having no effect of the body weight of the animals in H group. The plasma glucose level was diminished to ~ 100 ± 20 mg/dl, while apparently the cholesterol concentration was not significantly affected.

AGE-collagen fluorescence in the vascular mesenteric bed

At 24 weeks of simultaneous hyperlipidemia-diabetes the AGE-collagen fluorescence represented 51.34 ± 2.0 AU /µg protein, while in hyperlipidemia was 27.68 ± 1.7 AU /µg protein. In vivo feeding of the hamsters with 3 mM L-arginine for 4 weeks diminished the fluorescence of AGE-collagen to ~ 35% in HD group and to ~ 17% in H group as shown in Table 1.

AGE-proteins fluorescence in the lens

Data in Table 1 show that administration of L-arginine for 4 weeks to either simultaneous hyperlipidemic-diabetic or to hyperlipidemic hamsters (at 24 weeks of disease/s) resulted in a 65-70% diminishment in AGE-proteins fluorescence.

Table 1: Effect of administration of 3mM L-arginine (for 4 weeks) on the fluorescence of AGE-collagen (vascular mesenteric bed) and lens AGE-proteins in HD and H hamsters

Group of animals	*AGE-collagen fluorescence /µg protein: mesenteric vascular bed*	*AGE-protein fluorescence /µg protein: lens*
H	27.68 ± 1.7	3.25 ± 0.1
H + L-Arg	22.9 ± 0.9	0.97 ± 0.09
HD	51.34 ± 2.1	4.25 ± 0.2
HD + L-Arg	33.58 ± 1.3	1.47 ± 0.1

DISCUSSION

The quantitative estimation of autocatalitically generated AGE-adducts by the fluorescence emission at 440 nm when excited at 370 nm reflects a group reactivity, without allowing the identification of the distinct chemically defined structures formed during the nonenzymatic glycation of proteins (31). Recently, the fluorometric approach was used to assess the levels of circulating, excreted and lens accumulating AGE-proteins in diabetic patients (29, 30). Based on the statistically significant correlation between AGE levels and lens autofluorescence, the noninvasive measurement of the latter was introduced (ex: 365 nm/em: 434 nm) as a screening test of the secondary complications in diabetic patients (30, 32).

The fluorometric estimation of AGE "classical" Amadori rearrangement products does not interfere with the fluorescence of lipid peroxidation products, such as malondialdehyde (ex: 390 nm/ em: 460 nm) and other lipid-derived adducts (ex: 356 nm/ em: 460 nm)(33-35).

We have performed extensive tissues delipidation prior to the fluorescence measurements (at ex: 370 nm/ em: 440 nm). The efficiency of lipoprotein removal was verified in separate SDS-PAGE experiments on lipid-free lenticular extracts that did not show any high molecular bands typical for apoB (data not shown).

The fluorescence AGE-adducts does not interfere with the AGE-forming intermediates arrising from the oxidative reactions ("glycoxidation") such as pentosidine (fluorometry at ex: 335 nm/ em: 385 nm). The latter were beyond the scope of the present study. It is known that

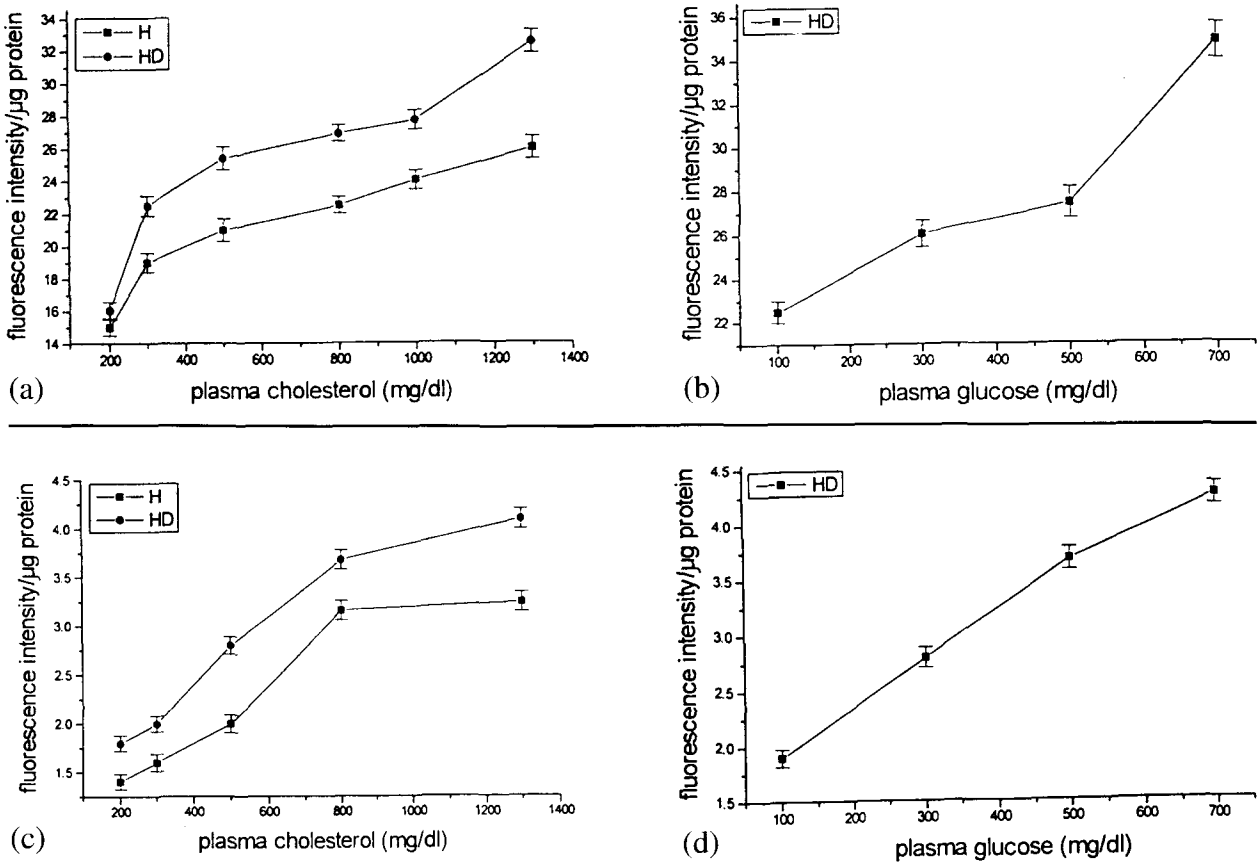

Fig. 3: Correlation between the AGE – protein fluorescence in tissues and the levels of plasma cholesterol and glucose of the simultaneous hyperlipidemic-diabetic (HD), and hyperlipidemic (H) hamsters. a, b: vascular mesenteric bed; c,d: lens.

AGEs are able of generating reactive oxygen species, but only in special conditions, including the interaction with receptors (36) and irradiation with UV-light (37) that predispose the proteins to further glycation (38).

The main finding of the present study is that the simultaneous insult of hyperlipidemia-diabetes (induced in hamsters for 24 weeks) generates an enhanced AGE-protein fluorescence of the vascular mesenteric bed and lens, correlated to the increase in circulating glucose and cholesterol concentrations (throughout the experiment). Interestingly, the increased fluorescence of AGE - proteins in the vascular mesenteric bed of hyperlipidemic - diabetic and hyperlipidemic hamsters may account for the impeded endothelium-dependent relaxation of the mesenteric arteries recorded by the myograph technique (39). AGE may form crosslinks responsible, at least in part, for the rigidity (stiffness) of the vascular wall, and are known to quench the dilatatory effect of the nitric oxide. The low fluorescence intensity of AGE-proteins in hamsters lens crystallins is in agreement with that of rat (22), and may be explained by the occurrence of a reduced number of constitutive proteins in this tissue.

In this study we had dissociated the effects of induced disease(s) from that of chronological age (which occurs simultaneously) by conducting two separate experiments: (i) on aging animals (6-48 weeks of age), and (ii) on induced hyperlipidemia-diabetes (2-24 weeks of disease, 12-36 weeks of age, as depicted by the scheme in Figure1. We, like others (19, 40) have found age-related increases in AGE-protein fluorescence.

Another finding of this study is that the augmentation of AGE-protein fluorescence in concomitant hyperlipidemic-diabetic hamsters is reduced by in vivo L-arginine administration (for 4 weeks).This effect is accompanied by other modifications, such as: an improvement in the weight loss, the restoration of plasma glucose concentration to normal levels, and the decrease in fluorescence of meseneric AGE-collagen. The improvement of the glycemic status of the diabetic animals after L-arginine administration may be related to its effect on insulin release from the pancreas (41). The effects of L-arginine on lowering the fluorescence of AGE- proteins may be explained by competition of exogenous arginine for the lysine-like residues involved in AGE-protein cross-linking (42). In addition, a protective role of L-arginine against the reactive oxygen species was recently reported (43).

Considered together, the results of this study may be of potential relevance in perception the occurrence, role, and modulation of AGE-proteins in patients with diabetes accelerated atherosclerosis.

EXPERIMENTAL PROCEDURES

Reagents

Streptozotocin, HEPES, and the enzymatic kits for glucose and cholesterol assays were obtained from Sigma (St. Louis , MO, USA). Chloroform, methanol, sodium hydroxide, and EDTA were from Merck (Darmstadt, Germany). Collagenase from Clostridium histolyticum was purchased from Boheringer Mannheim GmbH (Germany). All other reagents used were of analytical grade.

Animals

Eighty male Golden Syrian hamsters were maintained in standard housing conditions, on a 14hr/10hr light/dark cycle, and provided rodent chow and water ad libitum.The experiments were performed in accordance with "Principles of laboratory animal care" (NIH publication no. 83-25, revised 1985).

Experimental Protocol

Studies on aging

To assay the influence of aging, 20 normal hamsters (6 weeks old) were investigated at 12, 22, 36 and 48 weeks of advanced biological age, as depicted in diagram of Figure 1.

Induction of hyperlipidemia associated with hyperglycemia, and of hyperlipidemia

To this purpose 60 hamsters (12 weeks old) were divided into two groups: (i) concomitant hyperlipidemics and diabetics (HD) fed a standard chow supplemented with 3% cholesterol and 15% butter, and injected i.p. with streptozotocin (Sz, 50mg/kg body weight) freshly solved in 50 mmol/l citrate buffer pH 4.5 (20), and (ii) hyperlipidemics (H) fed the same fat-rich diet (15). No insulin was administer to the HD group to correct hyperglycemia. As mentioned in Figure 1, the studies were performed at 2, 6, 8, and 24 weeks since the induction of disease(s). Age-matched normolipidemic and normoglycemic hamsters were used as controls (C) throughout the experiment.

In vivo chronic administration of L-arginine

At 24 weeks of simultaneous hyperlipidemia - diabetes or hyperlipidemia only, 15 hamsters of each group were additionally fed for 4 weeks (44) with 125 µl 3 mM L-arginine in saline (45) introduced daily by gavage.

Plasma assays

Blood was collected on EDTA (2.7 µmol/l) from the venous retroorbital plexus (of slightly anesthetized animals) or from the left ventricle of the heart (at the sacrifice). Plasma was isolated, and the glucose and cholesterol concentrations were assayed spectrophotometrically with the Sigma enzymatic kits, as indicated by the manufacturer.

Collection of tissues

At the experimental time points, the hamsters were sacrificed by cervical disslocation. After laparotomy, part of the small intestine (7-15 cm from the pylorus) was removed, and the mesentery exposed. In order to obtain a sufficient amount of tissue for biochemical assays the whole mesenteric bed was dissected including arteries, veins and connective tissue.

After eyes removal, they were enucleated, slit at the limbus, and the lens isolated.

Processing of tissues

We were aware of the fact that the vascular mesenteric bed includes adventitial layers enriched in lipids, and that the lens contains high cholesterol and cholesterol oxides concentrations (46, 47).Therefore, we performed an extensive lipid removal from the tissues in a two step procedure: (i) 15 min in acetone (twice), and (ii)17hr in a chloroform/ methanol mixture (2/1, by volume). Subsequently, the lipid-free samples were solubilised in alkali as described (22). The extracts were sedimented by 5 min centrifugation at 7428g , and the clear supernatant used for: (i) protein assay, and (ii) measurement of the fluorescence of AGE-adducts.

Protein concentration assay

The Bradford method (48) was used to quantitate the protein concentrations, using bovine serum albumin as standard.

Measurement of AGE-proteins

Samples were brought to 0.5 µg protein/µl in 1N NaOH and the intensity of fluorescence at Ex: 370 nm, Em: 440 nm (4, 11, 32, 33) was detected with a Shimadzu RF-5001 PC spectrofluorimeter (Japan). The results were expressed in arbitrary units of fluorescence intensity (AU)/µg protein.

Isolation of AGE-collagen

The protocol reported by MacDonald et al. (19) was used to isolate the collagen from the protein extracts in the mesenteric vascular bed. Briefly, the tissue was cutted in small pieces, suspended in 0.1M phosphate buffer (pH 7.4), homogenized for 30s, and sedimented for 15 min at 1300g. The pellet was washed by resuspending in distilled water, and further defatted in chloroform/methanol mixture (2/1 by volume) at 4°C for 24hr. The sample was spun down for 15 min at 1300g, the pellet extracted in ice-cold methanol, washed by distilled water, and finally suspended in 0.02 mol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.5. The Clostridium histolyticum collagenase (250 U) was subsequently added in order to digest the collagen of the samples. The blank was prepared of the same amount of enzyme in 0.02 mol/l HEPES buffer, only. The enzymatic digestion of the samples was carried out at 37°C for 17hr, followed by centrifugation at 10,000g for 15 min at 4°C. The supernatant was used for the assay of: (i) protein concentration (as above), and (ii) AGE-

collagen, by fluorescence spectroscopy at 370/440 nm (11, 32, 33). The measurements for AGE-collagen (corrected against the collagenase blank) were expressed as arbitrary units of fluorescence intensity (AU)/μg protein .

Statistical analysis

Data were analyzed by Student test and one - way ANOVA to compare mean levels across the groups. The diagrams were plotted using the computer programs Harvard Graphics and Origin.

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ABBREVIATIONS

Advanced glycation endproducts: AGE
hyperlipidemic-diabetic: HD
hyperlipidemic: H
normal (control): C
low density lipoprotein: LDL
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: HEPES
arbitrary units of fluorescence intensity: AU

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